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Alphavirus nsP3 Functions To Form Replication Complexes Transcribing Negative-Strand RNA

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The alphavirus mutant Sindbis virus HR *ts*4, which has been assigned to the A complementation group, possessed a selective defect in negative-strand synthesis that was similar although not identical to that observed for the B complementation group mutant *ts*11 (Y.-F. Wang, S. G. Sawicki, and D. L. Sawicki, J. Virol. 65:985–988, 1991). The causal mutation was identified as a change of a C to a U residue at nucleotide 4903 in the nsP3 open reading frame that predicted a change of Ala-268 to Val. Thus, both nsP3 and nsP1 play a role selectively in the transcription of negative strands early in infection. The assignment of the mutation carried by an A complementation group mutant of Sindbis virus HR to nsP3 was unexpected, as mutations in other A complementation group mutants studied to date mapped to nsP2. Another mutant with a conditionally lethal mutation, *ts*7 of the G complementation group, also possessed a causal mutation resulting from a single-residue change in nsP3. Negative-strand synthesis ceased more slowly after a shift to the nonpermissive temperature in *ts*7- than in *ts*4-infected cells, and *ts*7 complemented *ts*11, but *ts*4 did not. However, the nsP3 of both *ts*4 and *ts*7 allowed reactivation of negative-strand synthesis by stable replication complexes containing nsP4 from *ts*24. Therefore, mutations in nsP3 affected only early events in replication and probably prevent the formation and/or function of the initial replication complex that synthesizes its negative-strand template. Because neither *ts*4 nor *ts*7 complemented 10 A complementation group mutants, the genes for nsP2 and nsP3 function initially as a single cistron. We interpret these findings and present a model to suggest that the initial alphavirus replication complex is formed from tightly associated nsP2 and nsP3, perhaps in the form of P23, and proteolytically processed and *trans*-active nsP4 and nsP1.

Sindbis virus, a member of the *Alphavirus* genus of the family *Togaviridae*, is an enveloped, positive-strand RNA virus that replicates in both vertebrate and invertebrate cells. The 49S genome of the heat-resistant strain of Sindbis virus (SIN HR) is 11,703 nucleotides (nt) in length, exclusive of the cap and poly(A) tract (48). After the virus enters the cytoplasm of the infected cell and uncoats, the genome is translated into two nonstructural protein (nsP) precursors, the polyproteins P123 and P1234, the latter by readthrough of an opal termination codon at the end of the nsP3 reading frame. Readthrough occurs at about a 20% efficiency, resulting in the underproduction of nsP4 polypeptides compared with nsP1, nsP2, and nsP3 (27). The P123 and P1234 polyproteins are proteolytically cleaved by a papain-like protease activity in nsP2 to yield the mature nsP1, nsP2, nsP3, and nsP4 or the fusion protein P34, which are numbered according to their gene order (7, 8, 18, 27). The nsP4 sequence contains the conserved GDD motif and functions as the viral polymerase (2, 12, 24, 25). The pattern of processing of the polyproteins changes at different times in infection, giving rise to different intermediate polyproteins (7, 17, 44). Once formed, the initial replication complexes synthesize genome-length negative strands that become templates for synthesis of the genome RNA and of a 26S subgenomic mRNA that is translated into the viral structural proteins.

During the alphavirus replication cycle, negative-strand synthesis occurs only at early times and requires simultaneous viral protein synthesis, whereas positive-strand synthesis, once started, is stable and continues even if protein synthesis is terminated (36, 40). Thus, there are two forms or activities of the alphavirus RNA-dependent RNA polymerase. We (36) had speculated that negative-strand synthesis was regulated by a mechanism utilizing one or more short-lived viral nsPs, which were modified by proteolytic cleavage, or some other post-translational activity. For instance, uncleaved or incompletely cleaved precursors of the nsPs would function in alphavirus negative-strand RNA synthesis but after cleavage would form the stable positive-strand polymerase. Experimental evidence supporting this notion has been obtained recently (25, 45).

We had studied the requirements for negative-strand synthesis with RNA-negative mutants of SIN HR that had been screened (40) for the rapid and selective loss of negative-strand synthesis after a shift to 40°C. Mutants *ts*4, *ts*6, and *ts*11 had been identified (40) as defective in continuing negative-strand synthesis under such conditions, although only *ts*4 and *ts*11 were defective specifically in negative-strand synthesis (37). The temperature-sensitive (*ts*) defect in *ts*11 was mapped to nsP1 (16, 49, 50), a protein that also possesses methyltransferase and guanylyltransferase activities (28). *ts*4, which had been assigned to subgroup II of the A complementation group (37), was of interest because, in addition to its being an RNA-negative mutant, it expressed a temperature-independent reduction in overall RNA synthesis and in the synthesis of subgenomic 26S mRNA relative to 49S genome RNA (20, 37). Although the mutations of the subgroup II A complementation group mutants have not been mapped, subgroup I mutants that are defective in polyprotein cleavage and 26S RNA synthesis carry mutations in nsP2 (16, 39). Therefore, we undertook a detailed characterization of *ts*4.

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MATERIALS AND METHODS

Viruses and cells. The mutants *ts*4, *ts*7, and *ts*11 of the heat-resistant strain of Sindbis virus (SIN HR) were isolated originally by Burge and Pfefferkorn (3, 4). A sample of *ts*4, obtained from Ellen Strauss, was plaque purified, and a stock was prepared following growth at 30°C in chicken embryo fibroblast (CEF) cells at a multiplicity of infection (MOI) of 0.1. A revertant of *ts*4 (*ts*4R) was isolated from a plaque that formed at 40°C and was plaque purified once at 30°C and once at 40°C before growth of a stock at 40°C. SIN *ts*7 is a complex mutant with two conditionally lethal mutations that affected plaque formation: a change of G to A at nt 3243, predicting a change of Asp-522 to Asn in nsP2, and a change of U to C at nt 5035, predicting a change of Phe-312 to Ser in nsP3 (16); only the Phe-312 to Ser change in nsP3 led to an RNA-negative phenotype. Toto1101:*ts*7B5 is a hybrid virus containing only the nsP3 mutation (16). It was produced from constructs of the infectious SIN cDNA clone (33) in which the *ts*7 region from nt 4845 to nt 5262 replaced the corresponding parental pToto1101 sequence.

Synthesis of cDNA and construction of recombinant viruses. The infectious SIN cDNA clone pToto1101 was used to create three deletion mutants of the parental sequence to enable rapid selection of recombinants. TEB14 is the deletion vector of pToto1101 lacking the region from nt 1406 to nt 2288 after digestion with *Eco*47III and *Bgl*II; TW20 lacks the region from nt 2713 to nt 4280 after digestion with *Cla*I and *Avr*II; and TSH6 lacks the region from nt 5114 to nt 6917 after digestion with *Sfi*I and *Hpa*I (see Fig. 2). Unless otherwise indicated, restriction and other enzymes were obtained from New England Biolabs (Beverly, Mass.). To generate the deletion vectors, pToto1101 cDNA was digested with pairs of restriction endonucleases, and the fragments were blunt ended with the Klenow fragment of *Escherichia coli* DNA polymerase (U.S. Biochemicals, Cleveland, Ohio) at 37°C for 15 min or with bacteriophage T4 DNA polymerase (U.S. Biochemicals) at 12°C for 15 min in buffer containing 80 μM deoxynucleoside triphosphates, purified by electrophoresis on low-melting-point agarose gels (FMC BioProducts, Rockland, Maine), and self-ligated with T4 DNA ligase (Promega, Madison, Wis.) in the presence of 50 μM ATP–20 mM dithiothreitol at 16°C for 4 h before transformation into MC1061 cells.

To construct recombinant viruses containing the various parts of the nsP genes from SIN *ts*4, purified *ts*4 virion RNA was reverse transcribed with Moloney murine leukemia virus RNase H^- reverse transcriptase (GIBCO BRL, Grand Island, N.Y.) in a reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 20 mM dithiothreitol by incubation at 42°C for 1 h. To obtain a first-strand cDNA copy of the nsP1-nsP2 region, oligonucleotide RH15-2 (nt 2811 to nt 2825; the gift of J. Strauss) was used as the first-strand primer; to obtain a first-strand nsP2-nsP3 cDNA, oligonucleotide YW15.6 (nt 5348 to nt 5366) was used as the first-strand primer. One-twentieth of the first-strand cDNA was then amplified by a PCR method modified for amplification of cDNA products larger than 1 kb (14, 51). As shown in Fig. 2, YH47, a positive-sense oligonucleotide containing the *Sst*I restriction site, the SP6 promoter sequence, and the 5′ 15 nt of the Sindbis genome was used as the second-strand primer with oligonucleotide RH15-2 to amplify the nsP1-nsP2 sequence, and oligonucleotide ID2 (nt 1366 to nt 1380, positive sense) was used as the second-strand primer with oligonucleotide YW15-6 to amplify the nsP2-nsP3 sequence. The reactions were carried out with AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.) in 10 μl of a solution containing 1× PCR buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2.5 mM $MgCl_2$, 0.1% gelatin) and the four deoxyribonucleotides, each at a concentration of 0.2 mM. The sample was heated for 1 min at 95°C before beginning the amplification cycle (1 min at 95°C for denaturation, 1 min at 43°C for annealing, and 5 min at 72°C for polymerization) for a total of 20 cycles followed by a final extension cycle of 30 min at 72°C. The products were electrophoresed through 0.8% low-melting-point agarose gels and purified with either phenol-chloroform or the GeneClean procedure (GeneClean II; Bio 101, Inc., La Jolla, Calif.). Following digestion with *Sst*I (GIBCO BRL) and *Cla*I or with *Bgl*II and *Spe*I and repurification of the fragments, the nsP1-nsP2 and nsP2-nsP3 *ts*4 cDNAs were ligated into the TEB14 or TW20 deletion vector, respectively, in place of the parental SIN sequence to create the hybrid clone pToto1101:*ts*4SC, which contained nt 1 to nt 2713 of *ts*4, encoding nsP1 and the N-terminal part of nsP2 coding region, and pToto1101:*ts*4BS, which contained nt 2288 to nt 5262 of *ts*4, encoding the C-terminal half of nsP2 and the N-terminal half of nsP3. For subcloning of pToto1101:*ts*4BS, the cDNA was digested with restriction enzymes that divided the sequence into two parts before ligation into the deletion vector TEB14 or TSH6 (see Fig. 2). Thus, the clone pToto1101:*ts*4BA contained the region from the *Bgl*II to *Avr*II site (nt 2288 to nt 4280), and pToto1101:*ts*4AS contained the region from the *Avr*II to *Spe*I site (nt 4280 to nt 5262).

The double mutant Toto1101:*ts*4:24R1 was created by combining in the same infectious pToto1101 cDNA clone the *ts*4 nsP3 mutation contained in the *Sst*I (nt −90)-*Spe*I (nt 5262) fragment of pToto1101:*ts*4AS and the *ts*24R1 nsP4 mutation that reactivated negative-strand synthesis at 40°C and was contained in the *Spe*I-*Sst*I fragment from pToto1101:24R1 (35). This strategy was possible because the nsP1-nsP3 (*ts*4) or nsP1-nsP4 (*ts*24R1) coding sequences of each genome had been assayed for phenotype, and regions other than those containing each of the two mutations gave wild-type phenotypes (35; this study). If there were any other undetected sequence differences between the mutant and SIN HR or Toto1101, such changes did not lead to *ts* virus production. Likewise, the double mutant Toto1101:*ts*4:11A1 contained both the *ts*4 nsP3 mutation and the *ts*11 nsP1 mutation and was created by ligating the *Cla*I (nt 2713)-*Aat*II (nt 7999) fragment of pToto1101:*ts*4AS in place of the parental sequence in pToto1101:*ts*11A1, which contains the *ts*11 nsP1 mutation at nt 1101 (16, 50). The presence of both mutations in each double mutant cDNA was confirmed by sequencing the cDNA clones and, for Toto1101:*ts*4:24R1 virus, by expression of new conditionally lethal phenotypes compared with the Toto1101:24R1 virus.

Sequencing and analysis of predicted protein changes. The hybrid cDNA was sequenced by the dideoxy chain termination method (34) with Sequenase 2.0 (U.S. Biochemicals). The *ts*4 and *ts*4R virion RNA was sequenced by the dideoxy method with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.). The parental and the predicted mutant nsP3 sequences were analyzed for secondary structural features (5, 10, 11), including folding, antigenicity, and hydrophobicity, with LaserGene sequence analysis software (DNASTAR, Inc., Madison, Wis.).

In vitro transcription and transfection of CEF monolayers. The plasmid cDNAs were linearized with *Xho*I restriction endonuclease before incubation with SP6 RNA polymerase for the preparation of transcripts that were used immediately thereafter for transfection of CEF by the DEAE-dextran method (33). Infected cells were maintained at 30°C in Dulbecco's modified Eagle's minimal essential medium (DMEM)

containing 5% fetal bovine serum until the development of cytopathic effect at about 36 to 48 h postinfection (p.i.), at which time the virus-containing medium was harvested.

Infection and viral RNA labeling. CEF monolayers in 35-mm or 60-mm petri dishes were infected with virus (MOI of 100) at 30°C. [^{3}H]uridine (50 μCi/ml for overall RNA synthesis or 200 μCi/ml for negative-strand RNA synthesis; ICN, Irvine, Calif.) incorporation into viral RNA was determined by pulse labeling in the presence of 20 μg of dactinomycin per ml at different times after infection and counting the acid-insoluble radioactivity in a fraction of the cell extracts. Where indicated in the text, infected cells were shifted to 40°C during the early phase of infection, at a time when about 10% of the maximum rate of RNA synthesis had been reached at 30°C, or were incubated continuously at 30 or 40°C. Cultures maintained at 30°C were labeled for 1-h periods beginning 1 h before duplicate cultures were shifted to 40°C. Cultures shifted to 40°C were pulse labeled for 30-min periods beginning at the time of the shift.

Determination of negative-strand RNA synthesis. Negative-strand RNA synthesis was determined by quantifying labeled negative-strand RNA in purified replicative form (RF) RNA, obtained by RNase A treatment of the [^{3}H]uridine-labeled viral replicative intermediates (RIs). The RF RNA was heat denatured and hybridized in the presence of an excess of unlabeled, purified 49S virion positive-strand RNA (41). A value of 50% indicates that 50% of the total labeled RNA was negative-strand RNA and 50% was positive-strand RNA. Because the RF is derived from RIs that are also active in positive strand synthesis, a value of 50% labeled negative-strand RNA also indicates that 100% of the total viral negative strands in these RIs were newly synthesized during the pulse period.

Complementation. Complementation tests were performed as described by Strauss et al. (46) and modified by Sawicki and Sawicki (37). Briefly, infected monolayers were washed with 40°C medium once at 1 h p.i. and again for three times at 4 h p.i. to remove unadsorbed virus. Virus released at 40°C was harvested at 7 h p.i. and quantified by plaque assay at 30°C. The complementation index was the ratio of the yield of virus from mixedly infected CEF cells, using each virus at an MOI of 20, at 40°C divided by the sum of the yields from cells infected at an MOI of 20 by each mutant alone.

RESULTS

SIN *ts*4 had a selective *ts* defect in negative-strand synthesis. Normally, alphavirus negative-strand RNA synthesis occurs within the first 5 to 6 h p.i. at 30°C and ceases thereafter. We confirmed that in *ts*4-infected cells, negative-strand synthesis ceased rapidly and within 30 min after a shift to 40°C at any time during the normal period of negative-strand synthesis. This observation is consistent with *ts*4's possessing a *ts* defect that selectively affects the production of negative strands (Fig. 1A). This was in contrast to positive-strand synthesis, which continued at 40°C but, as observed for all RNA-negative mutants of Sindbis virus, never increased to the maximal rate observed at 30°C (Fig. 1B). Revertants of *ts*4 (*ts*4R) occurred at a frequency of 1×10^{-5} to 6×10^{-5}, which was consistent with reversion of a single point mutation, and were found to lack the *ts* defect in negative-strand synthesis (data not shown).

ts4 mutant had a single mutation in the nsP3 coding region. Although the reversion frequency of *ts*4 predicted a single point mutation, we took into consideration the possibility that *ts*4 possessed mutations in both nsP1 and nsP2 because the complementation pattern of *ts*4 was complex (16, 37). Moreover, the assignment of *ts*4 to the A complementation group did not rule out the possibility of its having a mutation in nsP3, because complementation between either of two nsP2 mutants belonging to the A complementation group and the nsP3 mutant *ts*7 of the G complementation group, while expected from earlier results, was not observed (16) and because *ts*18, assigned originally to the G complementation group, turned out to be an nsP2 mutant (16). Therefore, we initially constructed two recombinant infectious cDNA clones to screen for mutations in nsP1, nsP2, and the conserved region of nsP3 of *ts*4. One contained all of the coding sequences of nsP1 and an N-terminal portion of nsP2 from *ts*4 (pToto1101:*ts*4SC; the *Sst*I-*Cla*I fragment); the other contained the remaining portion of the coding sequences of nsP2, only slightly overlapping the pToto1101:*ts*4SC sequences, and the coding sequences for the conserved region of nsP3 (pToto1101:*ts*4BS; the *Bgl*II-*Spe*I fragment) in place of the corresponding regions of the parental cDNA clone pToto1101 (Fig. 2). Use of the unique *Spe*I site at nt 5262 facilitated cloning and allowed assessment of the conserved N-terminal portion of the nsP3 sequence (nt 4101 to 5747) (48). Because the C-terminal portion of nsP3 can be deleted without significantly affecting transcription (23), it was unlikely that the conditionally lethal mutation of *ts*4 would reside in this part of nsP3. Following transcription and transfection of the recombinant RNA into CEF cells, the resulting hybrid viruses were analyzed for *ts* growth, for reversion frequency, and for their ability to continue negative-strand synthesis after a shift to 40°C early in infection.

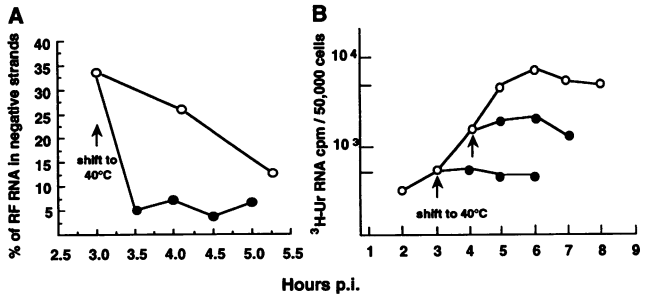

FIG. 1. Kinetics of *ts*4 RNA synthesis. (A) Negative-strand synthesis. SIN *ts*4-infected CEF monolayers were maintained at 30°C (○) or shifted to 40°C at 3 h p.i. (●) and incubated in medium that contained 200 μCi of [^{3}H]uridine (^{3}H-Ur) and 20 μg of dactinomycin per ml at the respective temperatures for 30-min periods at 40°C or for 60-min periods at 30°C. Cultures were harvested at the end of the pulse period, and their content of labeled negative-strand RNA was quantitated as described before (40). (B) Positive-strand synthesis. SIN *ts*4-infected cells were maintained at 30°C (○) or shifted from 30 to 40°C at 3 or 4 h p.i. (●), pulse labeled with [^{3}H]uridine for 30-min periods at 40°C or for 60-min periods at 30°C, harvested at the end of the pulse period, and analyzed for total acid-insoluble radioactivity, greater than 95% of which was in 49S and 26S positive-strand RNAs.

As shown in Table 1, only Toto1101:*ts*4BS, which is the hybrid virus that contained the *Bgl*II-*Spe*I region, was *ts* for growth, for virus production, and for negative-strand synthesis (i.e., it ceased negative-strand synthesis within 30 min after a shift to 40°C early in infection). The *Bgl*II-*Spe*I region was subcloned to identify the region containing the mutation. Toto1101:*ts*4BA (*Bgl*II-*Avr*II) had a wild-type phenotype, but Toto1101:*ts*4AS (*Avr*II-*Spe*I) was *ts* for virus production and for negative-strand synthesis (Table 1). Thus, the *ts*4 mutation was located between nt 4280 and nt 5262 and was within the open reading frame for the conserved portion of nsP3.

Two changes from the parental Toto1101 sequence were

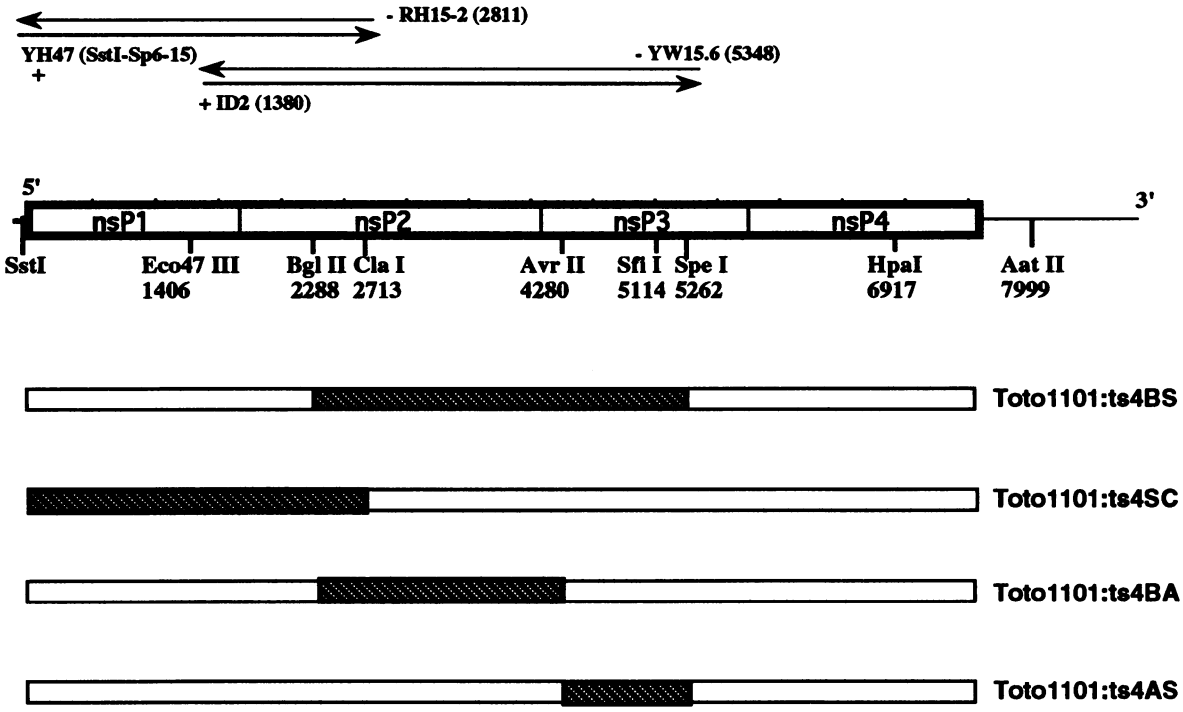

FIG. 2. Strategy for mapping the responsible mutation in *ts*4. Infectious cDNA clones that contained parts of the *ts*4 nsP coding regions in place of the corresponding regions of parental pToto1101 were made as described in Materials and Methods. Unique restriction endonucleases and their cleavage sites are marked under the schematic of the viral nsP1 through nsP4 coding regions; primers and deletion vectors are indicated above the schematic. The hatched regions represent sequences from *ts*4; the open regions are those from pToto1101.

found when the region from nt 4280 to nt 5262 of Toto1101:*ts*4AS was sequenced (Fig. 3). One was a change of a C to a U residue at nt 4903 that would change the Ala-268 in nsP3 to a Val; the second was a change of a U to a C residue at nt 5060 that would not alter the coding of the Val-320 residue and would be silent (Fig. 3). The same two base changes were present in the original *ts*4 genomic RNA purified from virions (data not shown). The *ts*4R genome retained the silent change at nt 5060 but had the parental C residue at nt 4903 and was thus a true revertant. As shown in Fig. 4, the predicted change of Ala-268 to Val in *ts*4 was in a region of nsP3 that is highly conserved among alphaviruses (12, 21, 43, 47). The other SIN HR *ts* RNA-negative nsP3 mutant is *ts*7 of the G complementation group, whose conditionally lethal mutation is a change of Phe-312 to Ser (16). The altered *ts*7 residue is located within the same highly conserved N-terminal half of nsP3 but 44

TABLE 1. Analysis of *ts*4 recombinant viruses

Virus	Fragment replaced (nt)	Growth[a]	Efficiency of plating (titer ratio, 40°C/30°C)	Negative-strand synthesis[b]
Toto1101		wt		wt
*ts*4		*ts*	1×10^{-5}–6×10^{-5}	*ts*
Toto1101:*ts*4SC	1–2713	wt	1.9×10^{-1}	wt
Toto1101:*ts*4BS	2288–5262	*ts*	2.0×10^{-5}	*ts*
Toto1101:*ts*4BA	2288–4280	wt	1.2×10^{-1}	wt
Toto1101:*ts*4AS	4280–5262	*ts*	2.3×10^{-5}	*ts*

[a] wt, wild type; *ts*, recombinants showing *ts* growth or a rapid, temperature-dependent cessation of negative-strand synthesis that mimicked that of the mutant *ts*4.

[b] The temperature sensitivity of negative strand synthesis was determined by shifting infected cultures to 40°C at 3 h p.i. or when the rate of positive-strand synthesis was 10% of the maximal rate and labeling with [^{3}H]uridine (200 μCi/ml) for 30-min periods before harvesting. The content of labeled negative-strand RNA in the isolated RFs was determined as described in Materials and Methods.

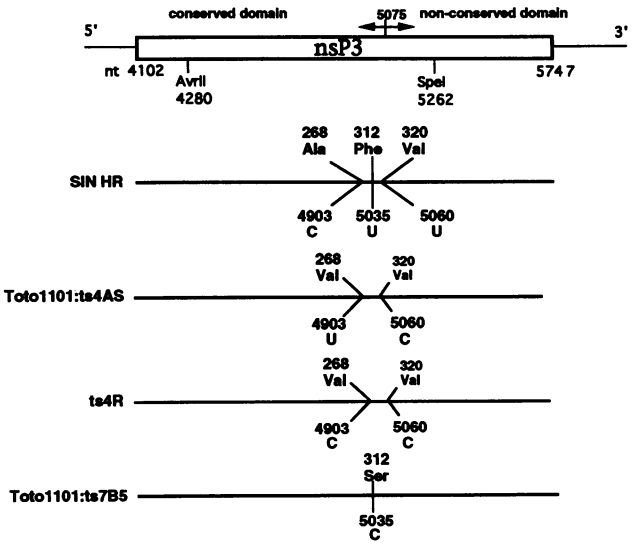

FIG. 3. Relative map positions of the predicted amino acid changes in nsP3 of Toto:*ts*4AS, *ts*4R, and Toto:*ts*7B5 compared with those in SIN HR and Toto1101. The data for Toto:*ts*7B5 were taken from Hahn et al. (16).

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                    260       270       280       290       300       310       320
SIN HR      257 PPKTLPCLCMYAMTPERVHRLRSNNVKEVTVCSSTPLPKHKIKNVQKVQCTKVVLFNPHTPAFVPA
SIN Ockelbo 257 ----------------------------------------Y-----------------------
SIN ts4     257 ----------V-----------------------------------------------------
SIN ts7     257 ----------------------------------------------------------S------
SFV         256 --R-V----R----A--IA----HQ--SMV----F----YHVDG----K-E--L--D-TV-SV-SP
RRV         256 ----V----R----A---A---M--T-AII----F----YR-EG----K-DR-LI-DQTV-SL-SP
ONN         256 ----V----R--------A---M-HTTSII----F----Y--EG----K-S-AL--DHNV-SR-SP
MID         254 --C-V----R-----------AAQ--QF-----F----Y--PG--R-A-SA-M---HDV--L-SP
VEE         261 --S------IH-------Q--KASRPEQI-----F----YR-TG---I--SQPI--S-KV--YIHP
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FIG. 4. Comparative amino acid sequences of alphaviral nsP3 proteins in the region of the *ts*4 and *ts*7 substitutions. The changes in SIN *ts*4 and SIN *ts*7 are outlined. Sequence data: SIN HR from Strauss et al. (48); Ockelbo from Shirako et al. (43); Semliki Forest virus (SFV) from Keränen and Kääriäinen (20); Ross River (RRV), O'Nyong-nyong (ONN), and Middleburg (MID) viruses from Strauss et al. (47); and Venezuelan equine encephalitis (VEE) virus, Kinney et al. (21).

codons downstream of the altered residue in *ts*4 (Fig. 4). We compared the phenotypes of the two nsP3 mutants to determine whether the two residues were part of the same functional domain.

Comparative analysis of SIN *ts*4 and *ts*7. In addition to two mutations in the nsP3 coding region, only one of which was conditionally lethal, the original *ts*7 mutant has a third mutation at nt 3247 in the nsP2 coding region that confers *ts* plaque formation but an $RNA^{+/-}$ phenotype (16). Therefore, we used the hybrid virus Toto1101:*ts*7B5 (16), which contained only the conditionally lethal U-to-C change at nt 5035 in the nsP3 coding region (Fig. 3). We confirmed that our stock of Toto1101:*ts*7B5 was *ts* for overall RNA synthesis and measured its efficiency of plating (the titer at 40°C divided by the titer at 30°C) as 7×10^{-5}. This was necessary to ensure that the phenotype that we observed was not due to leakiness, as reported (16) for the *ts*7B3 recombinant that contained both nsP3 base changes and had a reversion frequency of 10^{-2} to 10^{-3}. When Toto1101:*ts*7B5-infected cells were shifted to 40°C early in infection, positive-strand synthesis continued but did not increase significantly above the rate at the time of the shift (data not shown). Thus, Toto1101:*ts*7B5 replication complexes formed at 30°C were stable and functioned at 40°C. However, such cultures prematurely shut off negative-strand synthesis (Fig. 5). The cessation of negative-strand synthesis in Toto 1101:*ts*7B5 occurred sooner than the normal cessation observed at the end of the early phase of infection in parental SIN HR- or *ts*4R-infected cells, neither of which was defective in negative-strand synthesis, but it was not as rapid as that observed for *ts*11 and *ts*4, two mutants that are selectively defective in negative-strand synthesis. The more rapid cessation of negative-strand synthesis after the shift to the nonpermissive temperature by *ts*11 and *ts*4 than by *ts*7B5 was reproducible and suggested the possibility that negative-strand synthesis was immediately inhibited in *ts*11- and *ts*4-infected cells.

Mutations of nsP3 affect the initial but not the stable replication complex. To identify the point in the pathway of negative-strand RNA synthesis at which *ts*4 nsP3 was defective, we monitored events required for formation of the initial replication complex active in negative-strand synthesis separately from those necessary for the transcription of negative-strand RNA once the stable replication complex had been formed. This was possible because a mutant nsP4 encoded by the genomes of SIN *ts*24 and its revertants 24R1 and 24R2 led to the reactivation at 40°C of negative-strand synthesis by viral replication complexes that had formed earlier and were active in positive-strand RNA synthesis at the time of the shift to 40°C (35, 42). We constructed double recombinant viral genomes that expressed both the *ts*4 nsP3 mutation and the *ts*24 nsP4 mutation, i.e., Toto1101:*ts*4:24R1, and in the same experiments compared this virus with a double recombinant virus expressing the *ts*11 nsP1 mutation and the *ts*24 nsP4 mutation (Toto1101:*ts*11:24R1). Cells infected with either a double mutant, an individual mutant, or parental Toto1101 were shifted to 40°C at 6 h p.i. and incubated for a further 2 h at 40°C in the presence of cycloheximide to prevent further protein synthesis and of [^{3}H]uridine to label newly synthesized RNA. The viral RIs, which contain all of the viral negative-strand RNAs, were converted to RFs by RNase treatment, and their content of newly synthesized negative-strand RNA was measured. Cells infected with 24R1 virus reactivated negative-strand synthesis at 40°C and had 5 to 13 times more labeled negative strands than cells infected with only the *ts*4 or *ts*11 mutant or with Toto1101 (Table 2). Although the individual mutants expressing mutant nsP1 or nsP3 protein ceased negative-strand synthesis similarly early in infection, their ability to reactivate negative-strand synthesis late in infection differed dramatically. The nsP3 double mutant Toto1101:*ts*4:24R1 fully reactivated negative-strand RNA synthesis similarly to 24R1, whereas the nsP1 double mutant Toto:*ts*11:24R1 was blocked in reactivation (Table 2).

We also analyzed the independently isolated double recom-

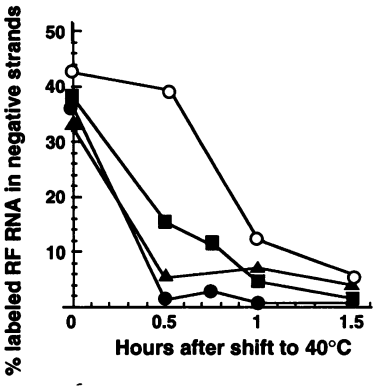

FIG. 5. Kinetics of cessation of negative-strand synthesis. Cultures were infected at an MOI of 100 at 30°C with nsP1 mutant *ts*11 (●), nsP3 mutant *ts*4 (▲), Toto1101:*ts*7B5 (■), or revertant *ts*4R (○) and maintained at 30°C until 3.5 h p.i., when they were shifted to 40°C. The continued synthesis of viral negative strands at 40°C was monitored by determining the amount of radiolabeled RNA in purified replicative structures (RFs) in 30-min pulse periods begun at the time of the shift to 40°C as described in Materials and Methods. The amount of newly synthesized negative-strand RNA is expressed as a percentage of the total labeled RF RNA in negative strands.

TABLE 2. Comparative analysis of the ability of nsP3, nsP1, and nsP2 mutants to effect reactivation of negative-strand synthesis

Expt no.	Virus	Mutant nsP	Negative-strand RNA[a] (cpm/10^4 RF cpm)
1	24R1	nsP4	2,010
	Toto1101		439
	*ts*4	nsP3	395
	Toto:*ts*4:24R1	nsP3 + nsP4	1,810
	*ts*11	nsP1	155
	Toto:*ts*11:24R1	nsP1 + nsP4	501
2	24R1	nsP4	2,590
	Toto1101		428
	*ts*7	nsP2 + nsP3	1,663
	Toto:*ts*7B5-3	nsP3	462
	Toto:*ts*7B5-7	nsP3	551
	Toto:*ts*7B5:24R1	nsP3 + nsP4	1,395
	Toto:*ts*7B5:24R1	nsP3 + nsP4	1,543

[a] Virus-infected cells were shifted to 40°C at 6 h p.i. and labeled at 40°C with [^{3}H]uridine from 6 to 8 h p.i. in the presence of cycloheximide. Viruses from two independent plaques of Toto:*ts*7B5 and from two separate cDNA clones of Toto:*ts*7B5:24R1 were tested. The viral RFs were obtained as described in Materials and Methods. The values are the averages of the RF RNA content of labeled negative strands from two to four duplicate cultures for each virus.

TABLE 3. Complementation analysis

Virus[a]	Mutant protein	Complementation index[b]				
		*ts*18	*ts*4	*ts*7	*ts*7B5	*ts*6
*ts*11 (B)	nsP1	5.20	1.60	3.00	5.2	76
*ts*15 (AI)		0.61		0.03		
*ts*17 (AI)	nsP2	0.04	0.60	0.22	1.9	300
*ts*18 (G)	nsP2		0.24	0.05		
*ts*21 (AI)	nsP2	0.12		0.37		
*ts*24 (AI)	nsP2	0.63	0.23			
*ts*133 (AI)	nsP2	0.63	0.04			
*ts*14 (AII)		0.71		0.06		
*ts*16 (AII)		1.08		0.38		
*ts*19 (AII)		0.07		0.19		
*ts*138 (AII)		0.07		0.18		
*ts*4 (AII)	nsP3		0.80		0.30	19.8
*ts*7 (G)	nsP3		0.12			
*ts*6 (F)	nsP4	2.40	19.80		24.8	

[a] The complementation group to which each mutant was originally assigned (4, 46) is shown in parentheses. The two subgroups into which the A complementation group mutants were placed subsequently are also indicated (37).

[b] Complementation values are the yield of the mixed infections divided by the sum of the yields of the infections with each mutant alone. Values of less than 2 are taken to indicate that complementation did not occur. Analysis was performed as described by Sawicki and Sawicki (37).

binant viruses containing the *ts*7 nsP3 mutation and the *ts*24 nsP4 mutation, i.e., Toto1101:*ts*7B5:24R1. This virus was found to be similar to Toto1101:*ts*4:24R1 and reactivated negative-strand synthesis (Table 2). Thus, nsP1 functioned in the transcription of negative strands by preformed or stable replication complexes as well as by newly created or initial replication complexes. Furthermore, the defect in nsP1 possessed by *ts*11 was expressed by mature and stable nsP1 proteins synthesized hours earlier and active in positive-strand synthesis at the time of the shift. On the other hand, the two mutations in nsP3 which were found in *ts*4 and *ts*7 prevented negative-strand synthesis early but did not affect the transcription of negative strands by preformed complexes late in infection. Because these two mutations in nsP3 affected only early functions, we interpret these results to mean that they affected only the initial replication complex during or immediately after its formation and did not affect the ability of the stable replication complex to recognize the promoter for negative-strand synthesis or the actual polymerization of the negative strands themselves.

Although the altered nsP3 of *ts*7 in Toto1101:*ts*7B5 lost negative-strand synthetic activity at 40°C (Fig. 5), the original *ts*7 reactivated negative-strand synthesis at 40°C by itself and in the absence of the 24R1 mutation (Table 2). Apparently this is due to the conditionally lethal mutation in nsP2 of *ts*7 that predicts a change of Asp-522 to Asn in the C region of nsP2, a region already implicated in subgenomic mRNA synthesis and polyprotein cleavage (16). This brings to three the number of separate *ts* alterations in the C region of nsP2 (Ala-517 to Thr [*ts*17], Asp-522 to Asn [*ts*7], and Asn-700 to Lys [*ts*133] [39]) that lead to reactivation of negative-strand synthesis at 40°C, two of which (*ts*17 and *ts*133) also confer *ts* 26S mRNA synthesis. The alteration of Asp-522 to Asn led to a level of negative-strand synthesis similar to that observed for each of the other two nsP2 mutants and half the level observed for the 24R1 nsP4 mutation (39). We interpret these findings to suggest that the nsP2 protein functions in some unknown manner, either directly or possibly indirectly, by affecting the activity of other essential factors, to influence promoter recognition for transcription of negative-strand versus positive-strand RNA by stable replication complexes.

We also determined whether both the *ts*11 nsP1 and *ts*4 nsP3 mutations were compatible in the same genome and whether their effects were additive. The presence of both mutations in the recombinant clones was verified by sequencing the double recombinant Toto1101:*ts*4:*ts*11A1 cDNA (data not shown). Transcription of the Toto1101:*ts*4:*ts*11A1 cDNA and transfection of the resulting RNA gave virus that had an efficiency of plating of less than 10^{-8}, consistent with the presence of two mutations. The Toto1101:*ts*4:*ts*11A1 virus continued positive-strand synthesis after a shift of the infected cells to 40°C early in infection but abruptly ceased negative-strand synthesis (data not shown). Thus, both mutations were tolerated in the same genome, and replication complexes containing both mutant proteins did not show detectably greater defectiveness than observed for replication complexes containing each mutant protein separately.

Complementation analysis of nsP3 and nsP2 mutants. Because intracistronic complementation was reported to occur with SIN mutants (16), we tested the ability of the two nsP3 mutants *ts*4 and *ts*7, which had been assigned to the A and G complementation groups (4, 46), respectively, to rescue each other and to complement mutations mapped to other nsPs. Complementation analyses (Table 3) showed that *ts*4 complemented the nsP4 mutant *ts*6 but not mutants encoding defective nsP1, nsP2, or nsP3 proteins. These findings were consistent with and extended our previous findings (37). Because *ts*7 contained a second *ts* mutation in nsP2 (16) and showed a minor defect in processing of P123 and P1234 polyproteins upon a shift to 40°C, leading to some accumulation of P23 (17), the nsP3 recombinant Toto:*ts*7B5 and *ts*7 were tested. Toto 1101:*ts*7B5 did not complement *ts*4 but did complement the nsP4 mutant *ts*6 and also the nsP1 mutant *ts*11, although weakly. However, not only *ts*7 but also Toto1101:*ts*7B5 failed to complement nine nsP2 mutants (five with mutations previously mapped to nsP2 and four subgroup II A complementation group mutants whose mutations were mapped only recently to

nsP2 [6a]) and *ts*15, whose phenotype predicts that its mutation will also map to nsP2 (37). Toto1101:*ts*7B5 and *ts*4 were not defective in polyprotein cleavage (data not shown) (37); thus, their failure to show complementation with A group mutants was not a result of the trapping of nonmutated nsPs to inactive precursors. In summary, virus with mutations in nsP4 and nsP1 complemented relatively efficiently viruses with mutations in nsP2 and nsP3, but none of 12 viruses with mutations in nsP2 and nsP3 complemented each other (Table 3). Finally, Toto 1101:*ts*7B5 showed a difference from *ts*4 in its ability to complement the nsP1 mutant *ts*11: the mutation at amino acid 268 in nsP3 of *ts*4 blocked complementation, while the mutation at amino acid 312 in nsP3 of Toto1101:*ts*7B5 did not. Our interpretation of these results is that nsP3 associates with nsP1 and a change in amino acid 268 of nsP3 affects this association, while a change in amino acid 312 of nsP3 does not. Such an association could occur at the level of the P123 polyprotein or after the cleavage and release of nsP1. We favor the latter interpretation because of the ability of the other nsP3 mutants, Toto1101:*ts*7B5 and *ts*7, to complement *ts*11's defective nsP1.

DISCUSSION

This study showed that a *ts* lesion resulting from a change of Ala-268 to Val in the nsP3 of SIN HR *ts*4 was responsible for the selective, rapid loss at 40°C of negative-strand synthesis, which was a phenotype that resembled the one possessed by the nsP1 mutant *ts*11 of the B complementation group of SIN HR mutants. Therefore, both nsP1 and nsP3 function in the synthesis of negative strands. The mapping of the mutation in a member of the A complementation group to nsP3 was unexpected, as the mutations in other A group mutants mapped to nsP2 (16, 37, 39); however, it paralleled the mapping of the mutations in the two SIN HR G complementation group mutants *ts*18 and *ts*7 to nsP2 and nsP3, respectively (16), and may have implications for the formation and structure of the early initial replication complex, as discussed below. Finding that nsP3 functioned in negative-strand synthesis was surprising because there is no analog of nsP3 in the plant viruses belonging to the SIN superfamily (19), which would need to form replication complexes synthesizing negative strands. SIN nsP3 is a 549-amino-acid phosphorylated protein whose N-terminal 325 amino acids are highly conserved among the alphaviruses, while the C-terminal 225 amino acids are variable or can be deleted (6, 21, 23, 32, 44, 47, 48). The N terminus of nsP3 contains essential sequences, since deletion of amino acids 218 to 273 is lethal (23, 26), as well as sequences that are highly conserved among the alphaviruses, rubiviruses, hepatitis E virus, and coronaviruses and flank their papain-like protease sequences (9, 12, 13). This latter "X" domain, which is located between amino acids 18 and 115 in SIN nsP3, has been suggested to function in polyprotein processing (1, 12, 13, 19). In the present study, amino acid substitutions in nsP3 at residues 268 and 312 did not drastically affect overall processing of polyproteins at 40°C (data not shown) (17, 20, 37) and there was no major loss of nsP4 functions that would have been expected if polyprotein processing had been affected (24, 25).

One difference that we observed between *ts*11 and *ts*4 was that the mutation in nsP1 of *ts*11 prevented the synthesis of negative strands by a stable replication complex, whereas the mutation in nsP3 of *ts*4 did not. We conclude that nsP1 functioned in negative-strand synthesis by both the initial or unstable form and the stable form of the replication complex, whereas nsP3 functioned in negative-strand synthesis only by the initial form of the replication complex. This could result if the mutation is nsP3 of *ts*4 caused a *ts* lesion that was expressed in the precursor of nsP3 that would be part of the initial but not the stable replication complex. On the other hand, the mutation in nsP1 of *ts*11 expressed itself both in the precursor and in the stable, final form of nsP1 and affected both the initial and stable replication complexes. The temporal requirement for functional nsP3 would not reflect its transient physical presence because nsP3 was a component of active replication complexes late in infection (2). Thus, the effect of the *ts* lesion in nsP3 of *ts*4 on early negative-strand synthesis could be related to its effect on P23 (*ts*) versus nsP3 (no effect). Of 63 SIN HR *ts* mutants (3, 4, 46) isolated after chemical mutagenesis, only the mutations in *ts*4 and *ts*7 mapped to nsP3. Comparison of the two nsP3 mutants showed that the Phe-312 to Ser change in the nsP3 of *ts*7 caused negative-strand synthesis to cease more slowly than the Ala-268 to Val change in the nsP3 of *ts*4. While *ts*7 failed to produce functional nsP3 or P23 proteins, it did produce nsP1 proteins capable of complementing the nsP1 mutant *ts*11, while *ts*4 did not. This could reflect a greater leakiness of the Phe-312 to Ser lesion or could indicate that residue 312 is near but not within the functional or folding domain of nsP3 that would be affected by the Ala-268 to Val mutation. Recently, two mutants with linker insertion mutations in the N-terminal 278 residues of nsP3 were found to resemble *ts*4 and *ts*7 in their RNA-negative phenotype, complementation pattern, and expression of a *ts* defect in negative-strand synthesis (22). Because they complemented *ts*11, we would predict that these mutants would resemble *ts*7, would cease negative-strand synthesis more slowly than *ts*11 and *ts*4, and would allow reactivation of negative-strand synthesis.

In *ts*4, the observed C-to-U transition is consistent with the original mutagenesis of the virus with nitrous acid (3). Although a change of Ala to Val might be considered minor since both are nonpolar (hydrophobic) amino acids and have similar isoelectric points (pI 6.0), a change of Ala to Val in the SIN E2 glycoprotein led to defective virion assembly (15). Computer analysis (5, 10, 11) of the protein sequence predicted that the Ala-268 to Val change would convert this region from alpha to beta structure (Chou-Fasman and Garnier-Robson analysis), decrease its hydrophilicity (Kyte-Doolittle analysis), and lower its probability of being on the surface (Emini analysis). The Phe-312 to Ser change, on the other hand, was predicted to increase hydrophilicity and create a short coil region. Such structural alterations in the nsP3 of *ts*4 could account for the reduced levels of overall RNA synthesis and for the change in the ratio of 26S subgenomic RNA relative to 49S genome RNA synthesis observed at the permissive temperature (20, 37), which are phenotypes that were not observed with *ts*7. The reduced level of overall viral RNA synthesis by *ts*4 at 30°C suggests that the region of nsP3 where the *ts*4 mutation is located is important for the formation of the initial replication complex. When synthesized and folded at 40°C, the *ts*4 mutated nsP3 is completely inactive. Once the complex had formed at 30°C, however, the mutation did not affect positive-strand synthesis by the stable replication complex at 40°C or prevent stable replication complexes containing the nsP4 of *ts*24 from reactivating negative-strand synthesis. The unusual aspect of the *ts*4 phenotype, compared with that of *ts*7, was the rapidity with which negative-strand synthesis ceased after exposure to high temperature. Several possibilities can be envisioned to explain these observations. The *ts*4 mutation could directly affect negative-strand synthesis by the initial replication complex or could act indirectly by altering the conformation and thus the activity of nsP1. In the latter case,

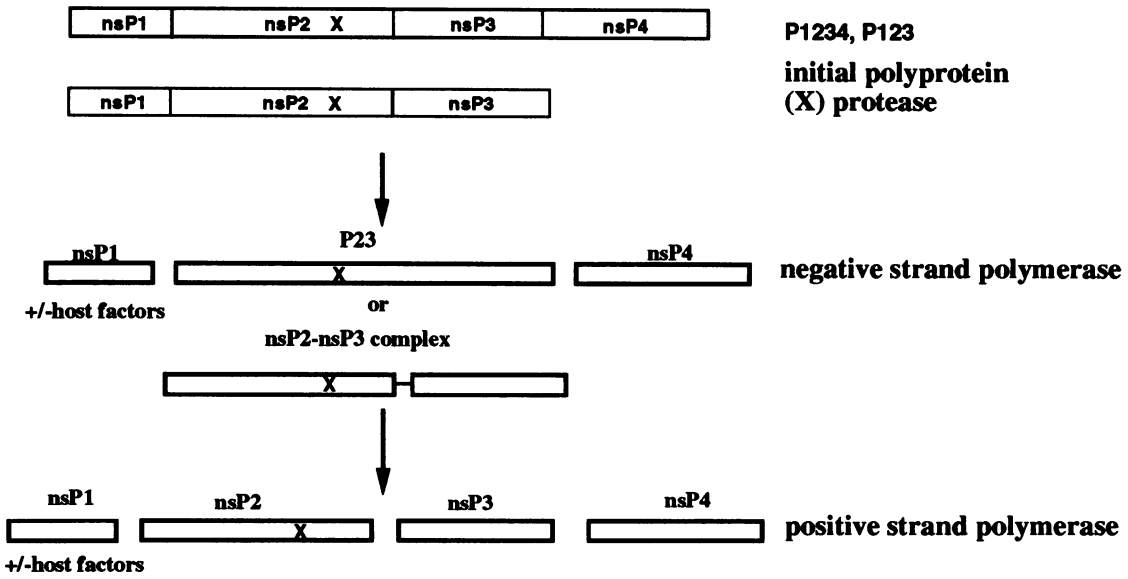

FIG. 6. Model for the processing of alphavirus polyproteins P1234 and P123 to form the negative-strand polymerase and its subsequent conversion to a positive-strand polymerase. The inability to detect complementation between 12 nsP2 and nsP3 mutants argues these two proteins may function as a *cis*-active complex to form a functional replication complex. Because their exact functional form is unknown, they are represented as a P23 fusion protein/polyprotein and as a *cis*-active nsP2-nsP3 complex. The X indicates the papain-like protease domain in nsP2 (7, 8, 13, 18).

nsP3 would play a role in the folding or modification of other nsPs.

Examination of complementation by SIN RNA-negative mutants was necessary to more fully assess the ability of the nsPs to dissociate, exchange, and assemble with each other and form replication complexes. Complementation analysis is more difficult when proteins are processed from a polyprotein and probably tend to associate with each other and not to diffuse and interchange. For example, alphaviruses other than SIN HR fail to show complementation and the SIN HR mutants show only low levels of complementation. The polyprotein strategy for the production of individual proteins that form a complex is an efficient mechanism to ensure that the proteins associate. Plant viruses belonging to the Sindbis superfamily produce homologous nsPs that actually function as polyproteins. In spite of these difficulties, the results of our complementation analysis confirmed the report by Hahn et al. (16), who found that *ts*7 (originally placed in the G group) did not complement *ts*18 (originally defined as a G group mutant but with its causal mutation mapped to nsP2) or *ts*24 (an A group mutant with a defective nsP2). Furthermore, we completed the analysis and found that this failure was a consistent property of all Burge and Pfefferkorn (3, 4) and Strauss and Strauss (46) nsP2 and nsP3 mutants. No nsP3 mutants complemented any of the nine nsP2 mutants, which have mutations mapping over at least 60% of the nsP2 protein, or *ts*15, which is a presumptive nsP2 mutant. This result argues that nsP4 and nsP1 were diffusable and active in *trans* and that their cleavage from the nascent P1234 and P123 polyproteins occurred early in the process of forming the initial replication complex. On the other hand, nsP2 and nsP3 would act as a single cistron because they formed a stable association immediately after their synthesis and presumably before their cleavage or because the polyprotein P23 was the functional form. Therefore, although there are four viral nonstructural proteins, there are actually only three complementation groups for the original SIN HR RNA-negative mutants. The only exception to this would be the recent report of two SIN linker insertion mutants carrying an extra six amino acids between residues 58 and 59 or 226 and 227 of nsP3 (22). The first mutant complemented *ts*18, which had a mutation in codon 509 for nsP2, but not *ts*17, which had a mutation in codon 517 for nsP2, while the latter mutant complemented both the nsP2 mutants but to a much lower extent than the nsP1 mutant *ts*11 and the nsP4 mutant *ts*6. Unlike single-residue substitutions, the addition of amino acid residues may distort and slow the folding of the nonstructural proteins and allow increased opportunity for complementation to occur. This would explain the high levels of complementation observed with these mutants. It would also argue that while a single cistron form like P23 may be functional, it is not required for the activity of replication complexes; nsP2 and nsP3 can also provide activity.

nsP4, not P34, is the functional form of the GDD replicase sequence (2, 24, 39), and its release from the P1234 polyprotein is necessary for transcriptional activation (24, 25, 45). Recent analyses of cleavage-defective variants of SIN demonstrated that fully cleaved nsP1, nsP2, nsP3, and nsP4 form replication complexes that are active in positive-strand synthesis (25, 45), while polyprotein P123 and nsP4 were active in negative-strand synthesis. These results support our discovery that replication complexes found late in infection contained fully processed nsPs (2). From the results presented here, we suggest that the initial replication complex that possesses negative-strand polymerase activity is composed of nsP4, nsP1, and P23 or tightly associated nsP2 and nsP3 (Fig. 6). The ability of *ts*11, which has a mutated nsP1 and is defective in negative-strand synthesis, to complement all other mutants except *ts*4 strongly argues that cleaved nsP1 can assemble into replication complexes that engage in negative-strand synthesis. Thus, cleavage of nsP1 from the polyprotein normally would be an early event. Moreover, a requirement for polyprotein P123 or P12 (17) is unlikely because cleaved nsP1 functions in negative-strand synthesis late in infection (35, 50). Thus, the current evidence suggests that, depending on conditions,

cleaved or uncleaved forms of nsP1, nsP2, and nsP3 plus cleaved nsP4 can assume the correct conformation and interact to function in negative-strand synthesis. Once formed, the short-lived nature of the negative-strand polymerase activity results from the conversion of the initial replication complex to a stable one that synthesizes positive strands by using the negative strand as a stable template (36, 38, 40). Conversion from the initial to the stable complex could be accomplished via cleavage of P23 and/or rearrangement of an nsP2 plus nsP3 complex. Others have proposed recently that a cleavage of P123 converts the replication complex into one possessing positive-strand polymerase activity (25, 45). Domains located to nsP2 and nsP4 (35, 39) affect the level of promoter recognition by the stable replication complex and determine the synthesis of positive strands relative to negative strands. Thus, the interaction of nsP2 and nsP4 may be affected by conformational changes that would result from mutations in nsP2 or nsP3. Although it had been suggested that the temporal synthesis of negative strands resulted from a regulated processing of the nsP polyproteins (17, 44), in particular from a change in the cleavage pattern of P1234 to form P34 and not nsP4 late in infection (7), much evidence now argues against this model (24, 25, 39, 45). Why negative-strand synthesis ceases early in infection is still unanswered. It may cease because of the failure to form P23 or P123, because of their more rapid cleavage late in infection, or because the exhaustion of a host factor(s) (2, 29–31) limits the formation of initial replication complexes late in infection.

Mutations in each of the four nsPs have been shown to give an RNA-negative phenotype. Although all alphavirus RNA-negative mutants fail to produce stable replication complexes that are active in positive-strand synthesis at 40°C, they do not share the distinct phenotype of *ts*4 and *ts*11, which is an immediate effect on negative-strand synthesis after a shift to the nonpermissive temperature. Therefore, other alphavirus RNA-negative *ts* mutants that are not defective in cleavage of the P1234 polyprotein and do not show as rapid a loss of negative-strand synthesis as *ts*4 and *ts*11 should show defects in the formation of replication complexes that occur after those caused by the *ts*4 and *ts*11 mutations. Such *ts* lesions would prevent the switch from negative- to positive-strand synthesis, i.e., the conversion of an initial to a stable replication complex. The ability of stable replication complexes containing nsP4 of *ts*24 to reactivate negative-strand synthesis has allowed us to discriminate between *ts* lesions that affect negative-strand synthesis specifically by the initial replication complex (*ts*4) from those that affect overall negative-strand polymerase activity (*ts*11). This has proven to be a powerful tool and will be especially useful in identifying mutants that are defective specifically in the formation of initial replication complexes, which requires negative-strand synthesis, or in the conversion of initial to stable replication complexes.

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